REMARKS

Claims 7-10 and 13-17 are all the claims pending in the application. Claims 7 and 13-17 are being examined. Claims 8-10 are withdrawn as directed to a non-elected invention.

Claim 7 has been amended to recite that the cell is transformed with an expression vector comprising a polynucleotide encoding a polypeptide, as supported, for example, at page 15, first through third full paragraphs. Claim 7 has also been amended to incorporate the recitation of claim 12, now canceled.

Claim 9 has been amended to correct an obvious clerical error.

Claim 13 has been amended to change the dependency from a canceled claim.

New claims 14-17 recite fewer than all of the members of the Markush group of claim 1 and are supported by original claim 1.

A. Rejection under 35 U.S.C. § 112, first paragraph

Claims 7 and 12-13 are rejected under 35 U.S.C. § 112, first paragraph as lacking written description support in the specification.

The Examiner contends that the specification "does not provide reasonable written description support for polypeptides of SEQ ID NO:2 or 4 in which 1 to 15 amino acids are deleted, substituted, or inserted, and additionally does not provide support for a method where the polypeptide has 80% or greater homology" to SEQ ID NO:2 or 4. The Examiner particularly notes that the specification provides no indication "as to which amino acid substitutions, insertions, or deletions are possible which maintain an ability too 'exhibit activity of promoting

insulin production by activation'." The Examiner mentions that "(t)he applicants do not provide even a single example of a polypeptide in which the amino acid sequence deviates from either SEQ ID NO:2 or NO:4."

For the following reasons, this rejection is traversed, respectfully.

The homology between the human polypeptide consisting of the amino acid sequence of SEQ ID NO:2 and the rat polypeptide consisting of the amino acid sequence of SEQ ID NO:4 is 80.6%, as described in the paragraph bridging pages 10 and 11 of the specification. That is, the amino acid sequence of SEQ ID NO:4 is disclosed in the present specification as an amino acid sequence having 80% or more homology with that of SEQ ID NO:2, and the amino acid sequence of SEQ ID NO:2 is disclosed in the present specification as an amino acid sequence having 80% or more homology with that of SEQ ID NO:4.

Furthermore, Applicants prepared Attachments A and B showing an alignment of the amino acid sequence of SEQ ID NO:2 (human sequence) and that of SEQ ID NO:4 (rat sequence), and another alignment of human, rat, and mouse sequences, respectively. Those skilled in the art would have known that alignment of such homologous sequences using any of the alignment algorithms well known in the art will generate a consensus sequence. One of skill in the art would have understood that a consensus sequence indicates the most common amino acid at each position and that regions of proteins containing strong consensus sequences typically represent conserved functional domains or structural elements. These conserved functional domains are often essential for the functioning of a protein, hence their conservation.

Accordingly, using alignment algorithms well-known in the art, one of ordinary skill in the art could gain an understanding as to what amino acids are likely to be essential for SEQ ID NO:2 or 4 to have activity of promoting insulin production by activation. Thus, the relationship between the amino acid sequence and the function of the protein is evident in view of the evolutionary conservation amongst homologues, and one of ordinary skill in the art would have been able to deduce, with a good degree of success, essential sequences common to members of the genus.

Accordingly, the Examiner is requested, respectfully, to reconsider and remove this rejection.

The Examiner's attention is also directed to new claims 14-17, which recite polypeptides (a), (b) and (c) or just (a) and (b).

B. Rejection under 35 U.S.C. § 112, second paragraph

Claims 7, 12 and 13 are rejected under 35 U.S.C. § 112, second paragraph as allegedly omitting "essential steps." The Examiner notes that:

-"activation" is not defined in claim 7;

-the claims do not provide how one would determine whether the polypeptide is activated or not; and

-there is no guidance for selection or confirming the activity of the selected substance.

For the following reasons, this rejection is traversed, respectfully.

Applicant submits that the term "activation" is definite to those skilled in the art from the description in the paragraph bridging pages 11 and 12 and the first full paragraph at page 13 of

the specification. According to the description in the specification, the G protein-coupled receptor of the present invention, is "activated" when a signal is transduced downstream of the G protein-coupled receptor (see first full paragraph at page 13).

Further, Applicant submits that there is sufficient guidance in the specification to confirm whether or not a polypeptide exhibits "an activity of promoting insulin production by activation." More specifically, from the paragraph bridging pages 11 and 12 and the first full paragraph at page 13 of the specification, it is apparent to those skilled in the art that a polypeptide in a cell transformed with an expression vector comprising a polynucleotide encoding the polypeptide is activated when the polypeptide promotes insulin production. The specification reads as follows:

> A method for confirming whether or not a polypeptide exhibits "an activity of promoting insulin production by activation" as used herein is not particularly limited, but it may be confirmed by, for example, the method described below, preferably a method described in Example 3. Namely, cells are respectively transformed with an expression vector comprising a polynucleotide encoding the polypeptide or a control expression vector without the polynucleotide, together with a plasmid in which a reporter gene (such as a luciferase gene) is linked downstream of an insulin promoter. After a predetermined number of hours (such as 24 hours) from the transformation, the medium is removed, the cells

are lysed with a cell lysing solution, and a reporter activity of each lysate is measured. When the reporter activity in the lysate of the cells transformed with the expression vector comprising the polynucleotide encoding the polypeptide (test cells) is increased, in comparison with that of the cells transformed with the control expression vector (control cells), it may be judged that the polypeptide exhibits the "activity of promoting insulin production by activation".

The state in which the polypeptide for a screening tool, a G protein-coupled receptor, is "activated" as used herein means a state in which a signal is transduced downstream of the G protein-coupled receptor regardless of a ligand binding. The polypeptide is activated when the total amount of an active form of G protein-coupled receptor exceeds a certain amount."

In view of the above remarks, the Examiner is requested, respectfully to reconsider and remove this rejection.

C. Rejection under 35 U.S.C. § 102(b)

Claims 7, 12 and 13 are rejected under 35 U.S.C. § 102(b) as anticipated by Fehmann et al. The Examiner relies on the doctrine of inherency, asserting that Fehmann et al. shows the testing of "several cAMP analogues to cause the same increase in insulin promoter activity" in

βTC-1 cells and noting that the specification provides that β cells express the polypeptides of SEQ ID NO:2 or 4. Thus, although the reference does not provide either sequence, the Examiner contends that the polypeptides are inherently present in the cells.

For the following reasons, this rejection is overcome.

The doctrine of inherent anticipation requires that the missing element is "necessarily" present in the asserted reference. As set forth in the MPEP in Section 2131.01 III. "[t]o serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill." *Continental Can Co. USA v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991).

In this respect, Fehmann et al. does not disclose or suggest a cell "transformed with an expression vector" comprising a polynucleotide encoding any one of the polypeptides (a) to (d), as now claimed.

Accordingly, the rejection is overcome and should be removed.

D. Rejection under 35 U.S.C. § 103(a)

Claims 7 and 12-13 are rejected under 35 U.S.C. § 103(a) as obvious over Bonini et al. (U.S. Patent No. 6,221,660), asserting that it discloses the sequences of SEQ ID NOs:2 and 4 and that the sequences are expressed in insulin-producing tissues that produce cAMP, a known activator of insulin promoter activity. Thus, the Examiner contends that it would have been

obvious to screen for compounds that activate SEQ ID NO:2 or 4 as a means of increasing insulin production.

For the following reasons, this rejection is overcome.

Bonini et al. disclose the amino acid sequence of SEQ ID NO:2 in the present specification (human SNORF25; SEQ ID NO:2 in Bonini et al.), and that of SEQ ID NO:4 in the present specification (rat SNORF25; SEQ ID NO:4 in Bonini et al.), as pointed out by the Examiner. Further, Bonini et al. list, as a use of agonists and antagonists of the SNORF25 polypeptides, treatment of many of the same diseases including diabetes with respect to both the agonists and antagonists (see column 26, lines 7-53). However, Bonini et al. do not have any support that the agonists or antagonists are, in fact, useful for treating these diseases. Further, the listed diseases to be treated with the agonists corresponds exactly to the list of diseases to be treated with the antagonists. That is, there is no teaching or suggestion to lead one of ordinary skill in the art in one of the other direction. Thus, the rejection is made in hindsight.

In addition, Bonini et al. disclose that an accumulation of cAMP was caused in response to retinoic acid (all-trans retinoic acid; ATRA), as pointed out by the Examiner, but do not disclose any data to support that retinoic acid can promote insulin production, i.e., insulin promoter activity. In this connection, Applicant submits herewith a copy of Clark et al. [Biochem. J. (1995) 309, 863-870] showing that retinoic acid suppressed insulin promoter activity (see Table 3). This reference was published prior to the priority date of the present application, and reported the above result, which is contrary to that disclosed in Bonini et al.

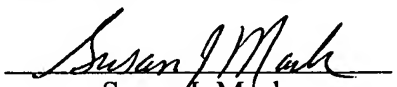
Therefore, as of Applicant's effective filing date the knowledge in the art was such that it did not teach whether agonists of the SNORF25 polypeptides promote insulin production.

In view of the above remarks, the Examiner is requested, respectfully, to reconsider and remove this rejection.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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23373
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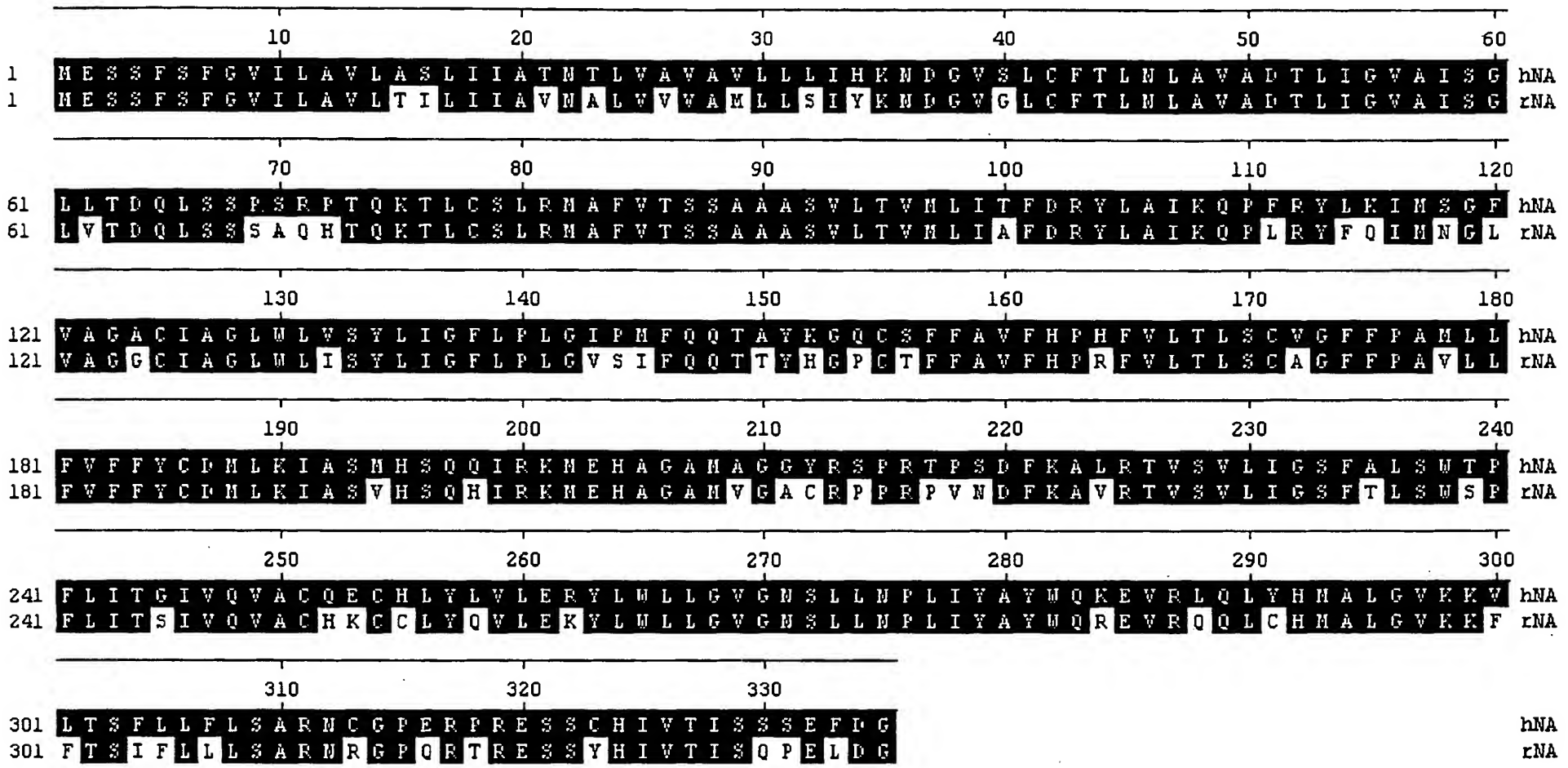
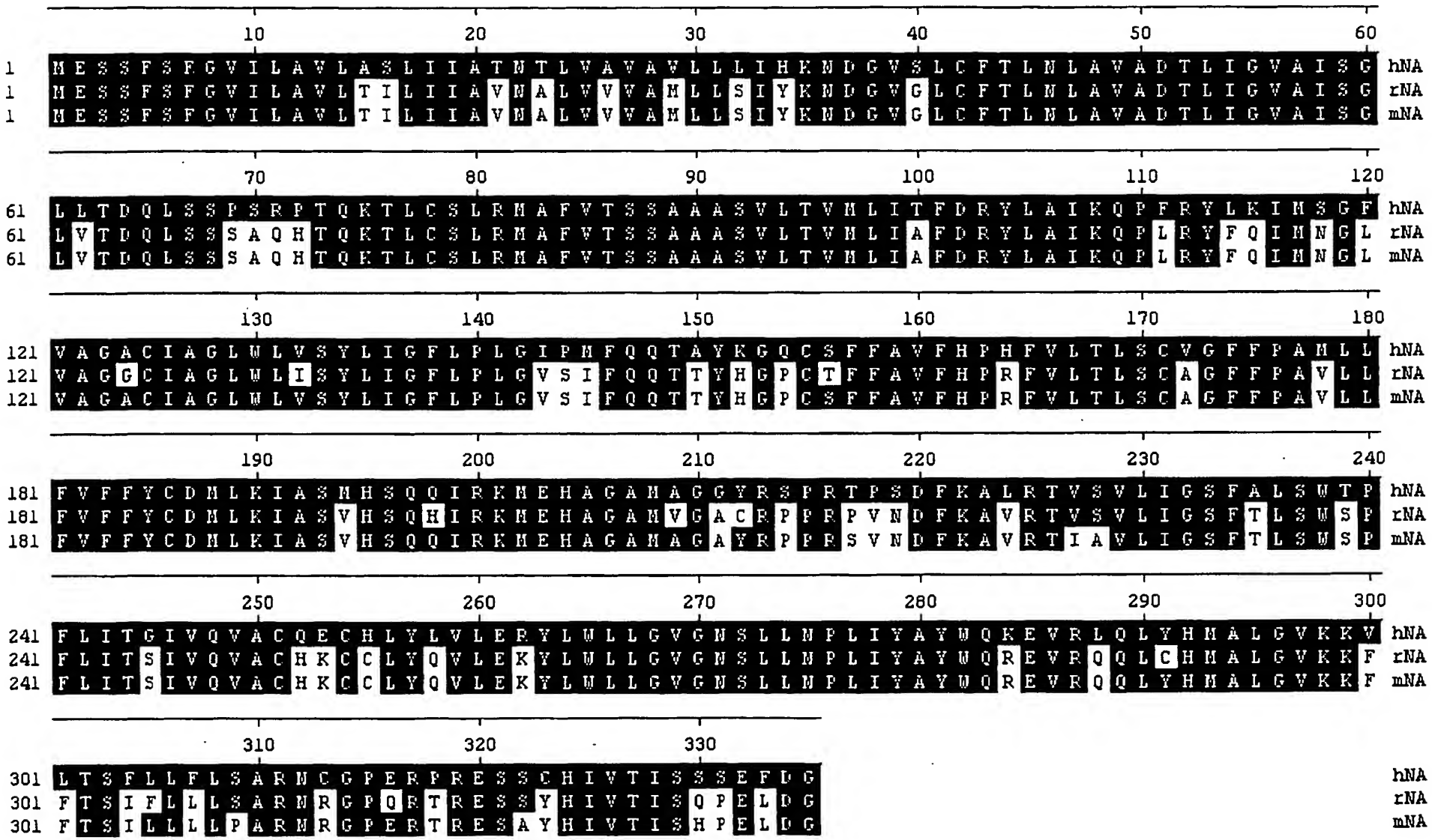
Date: August 16, 2007

Attachment A



Attachment B



Biochem. J. (1995) 305, 863–870 (Printed in Great Britain)

Identification and characterization of a functional retinoic acid/thyroid hormone-response element upstream of the human insulin gene enhancer

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A deletion analysis of the human insulin gene extending to 2 kb upstream of the transcription start site provided evidence of regulatory sequences located upstream of the insulin-linked polymorphic region (ILPR). Within this ILPR-distal region is a sequence (Ink, for insulin kilobase upstream) which contains three potential nuclear hormone-receptor half-sites, closely matching the consensus sequence AGGTCA. These sequences are arranged as a palindromic element with zero spacing overlapping a direct repeat with 2 bp spacing. The Ink sequence was used in electrophoretic mobility-shift assays within nuclear extracts from COS-7 cells overexpressing the vitamin D, thyroid hormone or retinoic acid receptors, or from an insulin-expressing hamster cell line, HIT-T15. These studies suggest that the insulin-expressing cell line contains thyroid hormone and retinoic acid receptors at least, and that these receptors are able to recognize the Ink sequence. Three copies of the Ink sequence were placed upstream of the thymidine kinase promoter and firefly luciferase reporter gene. In COS-7 cells expressing the appropriate nuclear hormone receptor, this construct was responsive to both thyroid hormone (18-fold) and all-*trans*-retinoic acid (31-fold). In HIT-T15 cells the same construct responded to all-*trans*-retinoic acid, but not to thyroid hormone. Within the context of a 2 kb insulin gene fragment, the Ink sequence was shown to be activated by retinoic acid and by the retinoic acid receptor, but acted as a negative element in the presence of both retinoic acid and the retinoic acid receptor. Mutagenesis studies demonstrated that the palindromic sequence was important for the retinoic acid response, and for binding of complexes containing retinoic acid receptor. In human islets of Langerhans, retinoic acid was shown to stimulate insulin mRNA levels. These results demonstrate that a functional nuclear hormone-receptor-response element is located upstream of the human ILPR. As retinoic acid and thyroid hormone are frequently involved in developmental regulatory processes, it is possible that this element may be important in the process of islet cell differentiation.

INTRODUCTION

The regulation of insulin gene transcription is dependent on the interaction of nuclear factors with DNA sequences located within a short distance upstream of the transcription start site [1]. In the human insulin gene a number of positively and negatively acting sequences have been mapped within a region up to −350 bp [2]. The helix–loop–helix factor insulin enhancer factor I [3] binds to an E box (consensus sequence CANNTG) located at −103 (the IEB1 box), and a second helix–loop–helix factor, upstream stimulating factor, binds along with several uncharacterized factors to a sequence at −230 (the IEB2 box) [4,5]. Another uncharacterized factor binds to the sequence GGGCCC adjacent to the IEB2 box [6]. A powerful negative regulatory element at −270 interacts with adjacent positive elements to modulate the activity of the insulin promoter, and a β-cell-specific factor, insulin upstream factor 1, binds to the sequence TCTAATG at three sites, −82 (the CT1 box), −215 (the CT2 box) and −319 (the CT3 box) [7].

The human insulin gene differs from most other insulin genes in that it contains a variable number of tandem repeats located upstream of −350 [8]. This variable number of tandem repeats [which is also known as the insulin-linked polymorphic region (ILPR)] contains repeats of the consensus sequence ACAGG-GGTCTGGGG, and is capable of adopting an unusual tetra-strand DNA structure [9,10]. Preliminary studies suggest that structural features within the ILPR may affect transcription of the human insulin gene [11,12]. Further support for a role for sequences upstream of −350 in regulating transcription of the gene comes from studies on transgenic mice [13].

The steroid-hormone receptors belong to a large family of transcription factors with representatives in organisms as diverse as man and *Drosophila*. The receptors for retinoic acid (RAR), thyroid hormone (TR) and vitamin D (VDR) belong to a subfamily recognizable on the basis of structural and functional similarities. They are localized in the nucleus in the absence of their ligands and can bind to DNA as homodimers; however, their binding affinity is greatly increased by heterodimerization with the retinoid X receptor (RXR) [14–17], the ligand of which is the 9-*cis*-stereoisomer of retinoic acid [18,19]. The response elements through which they regulate transcription are closely related, most commonly containing two copies of a half-site with the consensus sequence AGGTCA or Pu(G/T)TCA. According to the '3–4–5' rule, RXR/VDR, RXR/TR and RXR/RAR heterodimers show preferences for direct repeats with a spacing of 3, 4 and 5 nucleotides respectively [20,21].

To determine whether the ILPR and upstream sequences affect transcription of the human insulin gene, we undertook a deletion analysis up to −1995. Here we report the results of this study, which led to the identification and characterization of a functional

Abbreviations used: RAR, retinoic acid receptor; RXR, retinoid X receptor; CAT, chloramphenicol acetyltransferase; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; VDR, vitamin D receptor; TR, thyroid hormone receptor; ILPR, insulin-linked polymorphic region; DMEM, Dulbecco's modified Eagle's medium; αMHC, α-myosin heavy chain; T_3, tri-iodothyronine; PARP, peroxisome-activated receptor protein.

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thyroid hormone/retinoic acid-response element at around −1 kb.

EXPERIMENTAL

Cell lines

The Simian Virus 40 (SV40)-transformed monkey kidney cell line (COS-7) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum. HIT-T15, a β-cell line derived from SV40-transformed syrian hamster islets of Langerhans, was grown in RPMI 1640 supplemented with 10% (v/v) foetal calf serum.

Human islets of Langerhans

Human islets of Langerhans were isolated from the pancreas of human organ donors. Before removal of the pancreas, full and informed consent was obtained from the next of kin. All procedures were carried out with the approval of the appropriate ethical committee. Briefly, the pancreatic duct was cannulated *in situ* and digestion achieved by intraductal infusion of collagenase. The islets were then separated from contaminant acinar tissue by centrifugation on discontinuous density gradient of BSA in a semi-automated system [22]. The purified islets were placed in RPMI 1640 medium containing 10% (v/v) foetal calf serum and supplemented with 400 units of sodium penicillin G/ml and 200 μg of streptomycin sulphate/ml, and cultured at 37 °C in a humidified atmosphere of O_2/CO_2 (95:5, v/v) for several days before use.

Synthesis of vectors

A unique *Hin*dIII site was created at position +112 (with respect to the transcription start site) in the vector pUins300, which contains a 5 kb genomic fragment spanning the human insulin gene [23]. To excise 5′ fragments of varying sizes we used this *Hin*dIII site in conjunction with an *Nco*I site (−879), an *Rsa*I site (−1274) or an *Xho*II site (−1995). The 5′ fragments were filled in using Klenow enzyme, and ligated into a filled *Hin*dIII site upstream of the chloramphenicol acetyltransferase (CAT)-encoding sequence in the vector pBC0 [24], generating pBCins-879, pBCins-1274 and pBCins-1995. Constructs pBCins-339, pBCins-279 and pBCins-258 have been described previously [2].

Luciferase-expressing constructs were based on the vector pTkpLuc, which contains a herpes simplex virus thymidine kinase promoter fragment upstream of the firefly-luciferase-encoding sequence. pInk3.TkpLuc was synthesized by cloning three copies of the Ink (which stands for insulin kilobase upstream) oligonucleotide in sense orientation at a *Sma*I site upstream of the thymidine kinase promoter in pTkpLuc. pInk.TkpLuc, pInkA.TkpLuc, pInkB.TkpLuc and pInkC.TkpLuc were synthesized by cloning single copies of wild-type or mutant Ink oligonucleotides in sense orientation at the *Sma*I site in pTkpLuc.

Transfections treatment and assays

Transfection of COS-7 cells was performed using the calcium phosphate co-precipitation method as described by Docherty and Clark [25], with exposure of cells to precipitation for 8 h. Transfection of HIT-T15 cells was also performed using the calcium phosphate co-precipitation method, with exposure of cells to precipitation for 6 h, the last hour in the presence of added chloroquine (100 μM). After removal of the precipitate and washing with PBS, HIT-T15 cells were subjected to glycerol shock with 15% glycerol in serum-free DMEM for 2 min, then washed once more with PBS. After transfection, both COS-7 and HIT-T15 cells were maintained in serum-free DMEM, supplemented with selenium/insulin/transferrin, with or without the addition of retinoic acid (1 μM) or thyroid hormone [tri-iodothyronine (T_3); 100 nM].

After 24–30 h of treatment, cells were harvested by repeated freeze–thaw cycles in 100 μl of 100 mM KH_2PO_4/1 mM dithiothreitol. CAT was assayed as described by Docherty and Clark [25]. To quantify luciferase activity, 20 μl of cell extract was combined with 350 μl of 30 mM glycylglycine, pH 7.8, containing 2 mM ATP and 15 mM $MgSO_4$ and luminescence was measured in a Berthold instruments model LB 9501 luminometer on injection of 100 μl of 30 mM glycylglycine, pH 8, containing 0.5 mM luciferin. β-Galactosidase activity was measured using the Bactolight reagent kit, according to the manufacturer's instructions. Luciferase activity was expressed relative to β-galactosidase activity.

Preparation of nuclear extracts

Nuclear extracts of HIT-T15 and GH3 cells were prepared as described [26], with the exceptions that the final extraction buffer contained 10% glycerol and protease inhibitors aprotinin (1 mM), leupeptin (1 mM), pepstatin (1 mM) and *p*-aminobenzoic acid (1 mM). To prepare extracts of COS-7 cells over-expressing nuclear hormone receptors, calcium phosphate transfections were performed in 14 cm culture dishes with 40 μg of pCDM8, pCDM8.T_3R (expressing the $T_3\beta$ receptor cDNA), pCDM8.RAR (expressing the RARα cDNA) or pCDM8.VDR (expressing the VDR cDNA). After growth for 48 h in DMEM containing 10% foetal calf serum, extracts were prepared by the 'nuclear miniprep' method [27].

Mobility-shift assay

The α-myosin heavy chain (αMHC) sequence is 5′-CTGG-AGGTGACAGGAGGACAGCAGCCCTGA-3′ and the RARβ sequence is 5′-GGGTAGGGTTCACCGAAAGTTCA-CTCG-3′, where the direct-repeat sequence is underlined. ^{32}P-labelled oligonucleotide probe (50 fmol) was incubated with 2–10 μg of nuclear protein extract at 4 °C in 25 mM Tris/HCl, pH 7.8, containing 88 mM KCl, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 0.25 μg/ml BSA, 7.5% (v/v) glycerol and 0.05 μg/ml poly(dI-dC). Complexes were resolved by non-denaturing PAGE at 4 °C. Gels were dried down and protein–DNA complexes visualized by autoradiography [26].

Northern-blot analysis

Human islets of Langerhans were cultured in serum-free RPMI medium containing 3 mM glucose for 48 h before treatment. All-*trans*-retinoic acid (1 μM) was added to the culture medium and the cells were harvested at 2, 16 and 60 h after treatment. Total RNA was then prepared [28]. RNA (5 μg/well) was subjected to electrophoresis in a 2% (w/v) agarose/formaldehyde gel, and blotted on to a Hybond N filter (Amersham). Filters were then hybridized to ^{32}P-labelled human preproinsulin cDNA or 18S rRNA probes, and processed for autoradiography.

RESULTS

To determine the role of sequences upstream of −350 in regulating transcription of the human insulin gene, deletion constructs, generated by using conveniently located restriction endonuclease sites, were placed within plasmids containing the reporter gene CAT. CAT activity was then measured in trans-

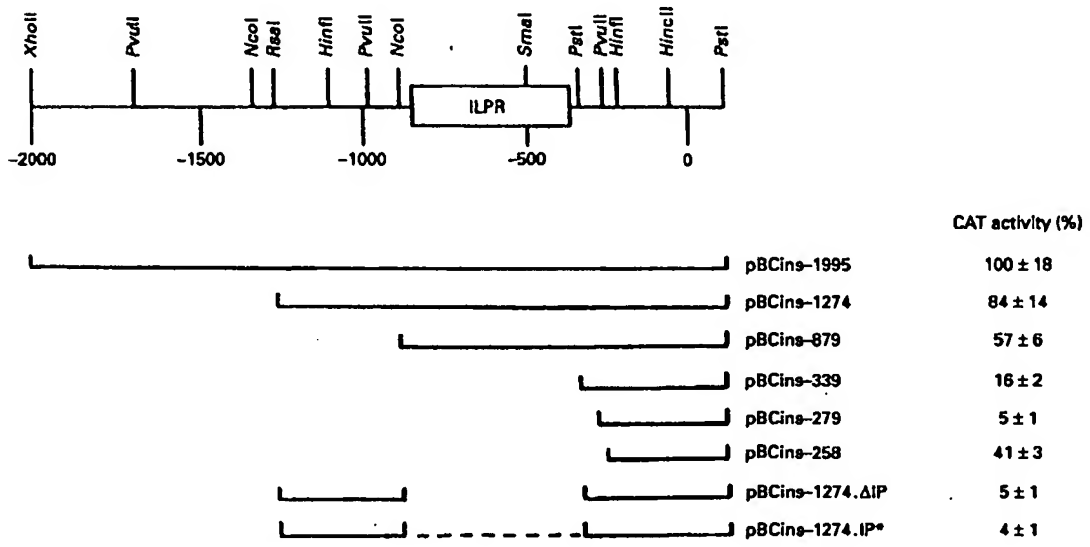

Figure 1 Deletion analysis of the human insulin gene 5′ region

HIT-T15 cells were transfected with the indicated recombinant plasmids, and CAT activity was assayed in cell extracts. CAT activity for each reporter plasmid is expressed relative to that for the plasmid containing the largest fragment of DNA (pBCins-1995). Results are expressed as means ± S.D. for three separate experiments.

fected HIT-T15 cells, and the results were expressed relative to the plasmid containing the largest fragment of DNA (pBCins-1995) (Figure 1). Deletion of sequences from −1995 to −1274 and −879 resulted in a stepwise decrease in CAT activity (to 84 and 57% respectively), suggesting the presence of positive regulatory elements within this region. The increase in CAT activity when sequences from −279 to −258 were deleted has previously been reported, and is due to the presence of a powerful negative regulatory element centred around −270 [2]. The deletion analysis also demonstrates the presence of a positive element between −339 and −279 which interacts with the adjacent negative element to modulate the activity of the promoter (A. R. Clark and K. Docherty, unpublished work).

Deletion of a 540 bp fragment containing the ILPR (pBCins-1274.ΔIP) dramatically decreased CAT activity. This was not simply the consequence of moving upstream sequences closer to the promoter, as replacement of the ILPR fragment with a similar-sized DNA fragment from bacteriophage λ (pBCins-1274.IP*) did not restore transcriptional activity. Although this result may indicate regulatory activity within the ILPR, this cannot at present be concluded with certainty because of the potential presence of regulatory elements between −879 and the 5′ boundary of the ILPR, or between −339 and the 3′ boundary of the ILPR. For instance, a high-affinity binding site for SP1 is present at −350 (A. R. Clark and K. Docherty, unpublished work).

Within the region −1274 to −879 we noted a cluster of three potential nuclear hormone-receptor half-sites, closely matching the consensus sequence AGGTCA. As illustrated in Figure 2, the cluster contains a palindromic element with zero spacing, overlapping a direct-repeat element with a 2 bp spacing (DR-2). The palindromic element very closely resembles the TREpal sequence, a much-studied artificial response element which mediates transcriptional activation by both thyroid hormone and retinoic acid. The direct-repeat element is closely related to retinoic acid response elements of the mouse cellular retinoid-binding protein (CRBPI) and the cellular retinoic acid-binding protein (CRABPII) gene.

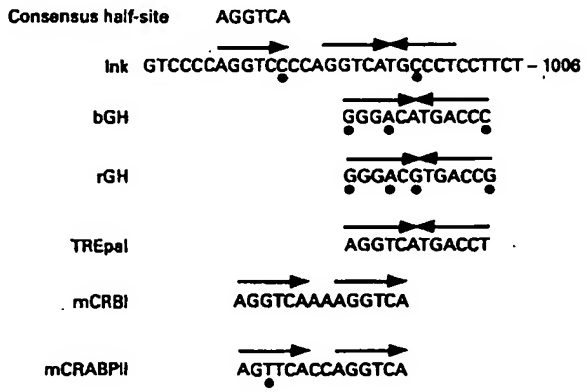

Figure 2 Ink sequence containing three potential nuclear hormone-receptor half-sites

The consensus sequence is a half-site for RAR, T_3R and VDR. The Ink sequence corresponds to −1037 to −1006 of the human insulin gene as cloned in plasmid phins300. Also shown are: (i) response elements in the bovine and rat growth hormone (bGH and rGH respectively) genes and TREpal (an artificial response element), which contain two receptor half-sites arranged as a palindrome with zero spacing, and which mediate responses to both retinoic acid and T_3; and (ii) direct-repeat retinoic acid response elements with 2 bp spacing (DR-2), which are located in the mouse CRBPI (mCRBPI) and the mouse CRABPII (mCRABPII). The black dots below each sequence denote variations from the consensus half-site sequence.

To determine whether this part of the human insulin gene 5′ region is able to bind members of the nuclear hormone-receptor family, we performed electrophoretic mobility-shift assays with an oligonucleotide probe (Ink, for insulin kilobase upstream),

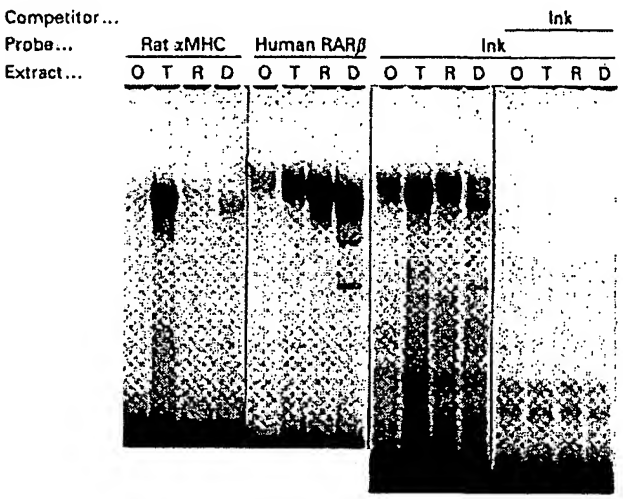

Figure 3 Binding of RAR T_3R and VDR receptors to the Ink sequence after expression of their cDNAs in transfected COS-7 cells

COS-7 cells were transfected with an empty expression plasmid pCDM8 (O) and with pCDM8 vectors containing cDNAs encoding the T_3R (T), RAR (R) and VDR (D). Nuclear extracts were prepared and used in electrophoretic mobility-shift assays with the following radiolabelled oligonucleotide probes containing: (i) a sequence from the rat αMHC gene 5′ region, which contains a functional T_3-response element; (ii) a sequence from the human RARβ gene 5′ region which responds to retinoic acid; and (iii) the Ink sequence. A competition experiment was performed using the Ink probe, in which the nuclear extracts were preincubated with a 50-fold excess of unlabelled Ink oligonucleotide before addition of the radiolabelled probe.

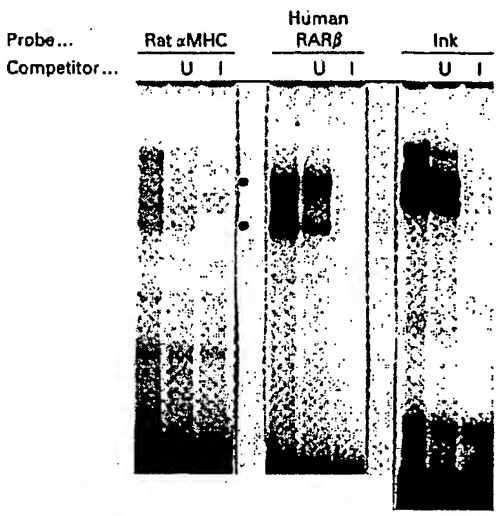

Figure 4 Demonstration that T_3R- and RAR-binding activity is present in HIT-T15 cells

Nuclear extracts from HIT-T15 cells were used in electrophoretic mobility-shift assays with radiolabelled oligonucleotide probes containing: (i) a sequence from the rat αMHC gene 5′ region, which contains a functional T_3-response element; (ii) a sequence from the human RARβ gene 5′ region which responds to retinoic acid; and (iii) the Ink sequence. Competitions were performed by preincubating the nuclear extracts with a 50-fold excess of an unrelated oligonucleotide (U) or with the Ink oligonucleotide (I). Using the rat αMHC probe, multiple bands were observed, two of which (indicated by the black dots) were specifically competed for by the Ink oligonucleotide.

the sequence of which is shown in Figure 2. As a source of nuclear hormone receptors we used COS-7 cells, which contain RXRs, but are thought to be devoid of T_3Rs, RARs or VDRs. Nuclear extracts containing specific members of the nuclear hormone-receptor family were generated by transfection of COS-7 cells with appropriate pCDM8-based expression vectors. As controls, we tested these extracts against well-characterized response elements: a sequence from the rat αMHC gene 5′ region that responds to T_3 but not to retinoic acid, and a sequence from the human RARβ gene 5′ region that is primarily responsive to retinoic acid. We also tested extracts against a vitamin D-response element of the rat osteocalcin gene; however, the binding of API to the element obscured interactions with the nuclear hormone receptors (not shown). As seen in Figure 3, the αMHC probe generated a strong complex only with the T_3R nuclear extract, although a minor novel complex was also observed with the VDR extract. In agreement with previous reports, little or no binding of RAR to this sequence was observed. In contrast, the RARβ probe generated the strongest complex with the RAR extract, with both T_3R and VDR extracts giving rise to weaker novel complexes. Although we have not been able to demonstrate binding of VDR to a natural vitamin D-response element, we believe that transfected COS-7 cells do generate VDR, as novel complexes appear on the αMHC and RARβ probes. When tested against the Ink sequence, the T_3R, RAR and VDR extracts each generated novel retarded bands, suggesting that this element can be recognized by complexes containing each of the three nuclear hormone receptors tested. The specificity of these complexes was demonstrated by self-competition with an excess of unlabelled Ink oligonucleotide. An unrelated oligonucleotide failed to compete (not shown).

Binding of nuclear factors to the Ink sequence was examined using an extract of the hamster β-like cell line HIT-T15 (Figure 4). For comparison, the αMHC and RARβ probes were again used, and parallel mobility-shift assays were performed with a nuclear extract of the pituitary cell line GH3, which contains T_3Rs, RARs and VDRs. With the αMHC probe, the β-cell extract generated several rather weak complexes, of which two (marked) were competed for by the Ink sequence, but not by an unrelated competitor. The RARβ probe generated two strong complexes, both of which were specifically competed for by the Ink sequence. The Ink sequence itself generated two very strong and specific retarded complexes, in addition to a third complex of lower mobility, which was partially competed for by the non-specific competitor. Despite differences in the precise positions of retarded complexes, the pattern obtained with a GH3 nuclear extract was similar (results not shown). Assuming that endogenous receptors possess the same binding specificity as previously demonstrated with transfected COS-7 cells (Figure 3), these observations strongly suggest that the β-like cell line contains receptors for both thyroid hormone and retinoic acid. With the rat osteocalcin vitamin D-response element both GH3 and HIT-T15 nuclear extracts generated complexes which could be competed for by the Ink sequence (not shown); however, it is not certain that these complexes contain VDRs rather than other members of the nuclear hormone-receptor family. The ability of the Ink oligonucleotide to compete for binding to αMHC and RARβ probes supports the conclusion that the Ink sequence is a natural binding site for members of the nuclear hormone-receptor family.

To determine whether the Ink sequence was functionally active, three copies of the Ink oligonucleotide were cloned in sense orientation upstream of the thymidine kinase promoter which regulates expression of the firefly luciferase reporter gene in the vector pInk3.TkpLuc. This construct was co-transfected

Table 1 Response of the Ink sequence to T_3 and retinoic acid in transfected COS-7 cells

COS-7 cells were co-transfected with plasmid pInk3.TkpLuc and an empty pCDM8 vector (pCDM8O) or pCDM8 containing cDNAs encoding the T_3R (pCDM8T) or the RAR (pCDM8R). The cells were then incubated in medium with no addition or containing T_3 (100 nM) or all-*trans*-retinoic acid (RA) (1 μM). Luciferase activity was then assayed in cell extracts and expressed relative to that measured in cells co-transfected with pInk3.TkpLuc and the empty pCDM8 vector. The results are from a single experiment and represent means ± S.D. ($n = 4$). Similar results were obtained in three separate experiments. ND, not determined.

	Relative luciferase activity		
Addition	pCDM8O	pCDM8T	pCDM8R
None	1.00 ± 0.12	0.99 ± 0.07	0.73 ± 0.13
T_3	1.05 ± 0.03	17.47 ± 0.87	ND
RA	6.51 ± 1.11	ND	30.84 ± 4.50

Table 2 Responsiveness of the Ink sequence to retinoic acid in transfected HIT-T15 cells

HIT-T15 cells were transfected with the plasmid pTkpLuc (pTkp) or pInk3.TkpLuc (pInk3Tkp). The cells were incubated in medium with no addition or containing T_3 (100 nM) or all-*trans*-retinoic acid (1 μM) (RA). Luciferase activity was then assayed in cell extracts and expressed relative to that measured in cells incubated in the absence of added hormone. The results are from a single experiment and represent means ± S.D. ($n = 4$). Similar results were obtained in three experiments.

	Relative luciferase activity	
Addition	pTkp	pInk3Tkp
None	1.00 ± 0.06	1.00 ± 0.05
T_3	0.93 ± 0.05	0.81 ± 0.01
RA	1.42 ± 0.07	4.10 ± 0.18

into COS-7 cells with pCDM8, or with a pCDM8-derived expression vector for $T_3R\beta$ or RARα. Subsequently cells were maintained in serum-free medium, or in serum-free medium supplemented with T_3 (100 nM) or all-*trans*-retinoic acid (1 μM) (Table 1). For comparison, similar transfections were carried out with the parental pTkpLuc vector, which contains the thymidine kinase promoter upstream of the luciferase-encoding sequence, and lacks any human insulin 5′ sequences. The control vector pTkpLuc was not regulated by any of the ligands tested, in either

Table 3 Effect of RAR and retinoic acid on pBCIns-1995 in transfected HIT-T15 cells

HIT-T15 cells were transfected with plasmid pBCIns-1995 on its own or along with plasmid pCDM8R. The cells were then incubated in medium with no addition or containing all-*trans*-retinoic acid (1 μM) (RA). CAT activity was then assayed in cell extracts and expressed relative to that measured in cells incubated in the absence of added hormone. The results are from a single experiment and represent means ± S.D. ($n = 4$). Similar results were obtained in four separate experiments.

	Relative CAT activity	
Addition	pBCIns-1995	pBCIns-1995 + pCDM8R
None	1.00 ± 0.30	2.62 ± 0.50
RA	1.73 ± 0.20	0.81 ± 0.31

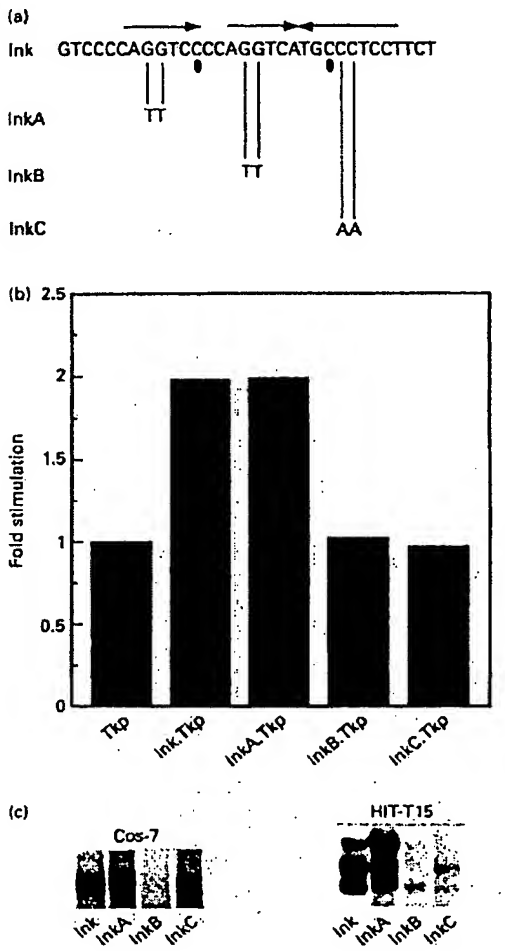

Figure 5 Sequence requirements for activation of the Ink sequence by retinoic acid

(a) The Ink sequence is shown along the mutant oligonucleotides that contain a 2 bp transversion mutation in the A motif (InkA), the B motif (InkB) and the C motif (InkC). (b) COS-7 cells were co-transfected with pCDM8R and pTkpLuc (Tkp), pInk.TkpLuc (Ink.Tkp), pInkA.TkpLuc (InkA.Tkp), pInkB.TkpLuc (InkB.Tkp) or pInkC.TkpLuc (InkC.Tkp), and the cells incubated in medium containing all-*trans*-retinoic acid (1 μM). Luciferase activity was then measured in the cell extracts and expressed relative to that in cells transfected with the plasmid pInk.TkpLuc. The results shown are representative of three separate experiments. (c) Electrophoretic mobility-shift assays using nuclear extracts prepared from COS-7 cells, which had been transfected with pCDM8R, or from HIT-T15 cells, and the indicated oligonucleotide probes containing the Ink, InkA, InkB or InkC sequence. Only the portion of the autoradiograph showing the specific retarded complexes is shown.

the presence or absence of their receptors (not shown). In the presence of the T_3R, the addition of T_3 brought about an almost 18-fold increase in reporter gene activity. A sixfold induction of transcription was observed with retinoic acid in the absence of the RARα expression vector. This might be explained by stereoisomerization of all-*trans*-retinoic acid to its 9-*cis*-form, which is a ligand for the RXRs present in COS-7 cells. In the presence of the RARα-expression vector, the induction by retinoic acid was 31-fold.

The activity of the Ink sequence was then tested in a β-cell line. HIT-T15 cells were transfected with pTkpLuc or with pInk3.TkpLuc and treated with T_3 or retinoic acid as above (Table 2). Only retinoic acid significantly increased the trans-

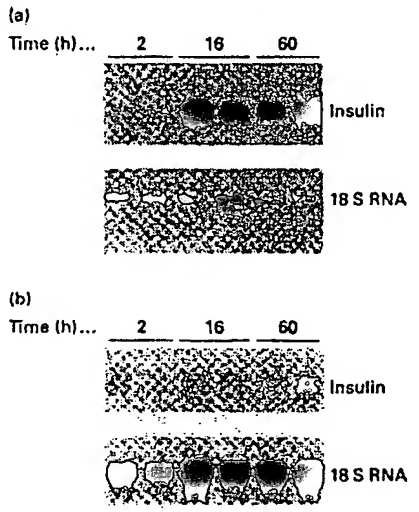

Figure 6 Effect of retinoic acid on insulin mRNA levels in human islets of Langerhans

Northern-blot analysis of total RNA extracted from human islets of Langerhans cultured for the indicated periods of time in the presence (a) or absence (b) of 1 μM all-*trans*-retinoic acid. Filters were hybridized with cDNA probes complementary to human preproinsulin and 18 S RNA as indicated. Similar results were obtained on three separate occasions.

criptional activity of pInk3.TkpLuc, and only approximately fourfold. Decreasing the quantity of reporter construct introduced into the HIT-T15 cells to 0.2 μg did not significantly alter this result (not shown). To determine whether the Ink sequence was responsive to hormone in the context of the 2 kb insulin gene enhancer fragment, plasmid pBCins-1995 was transfected into HIT-T15 cells alone or in combination with pCDM8R. Retinoic acid on its own caused a 1.7-fold stimulation of CAT activity. In the absence of ligand, the receptor (RAR) caused a 2.62-fold increase in CAT activity; however, in the presence of retinoic acid and its receptor CAT activity was suppressed to 31% of that seen in the presence of receptor alone (Table 3). The construct pBCins-879, which lacked the Ink sequence, was unresponsive to RAR in the absence or presence of retinoic acid (results not shown).

We next examined the sequence requirements for transcriptional activation by retinoic acid. As illustrated in Figure 5(a), the Ink element contains three close matches to the AGGTCA consensus half-site for binding of nuclear hormone receptors. The first of these (A motif) diverges from the consensus only at the sixth position, the second (B motif) is a perfect match and the third (C motif) diverges only at the fourth position. To investigate the involvement of these three half-sites in binding of RAR and transcriptional induction by retinoic acid we synthesized variants of the Ink oligonucleotide containing 2 bp transversion mutations in the A motif (InkA), the B motif (InkB) or the C motif (InkC). These, and the wild-type Ink oligonucleotide, were cloned in single copy upstream of the thymidine kinase promoter in pTkpLuc, generating the constructs pInk.TkpLuc, pInkA.TkpLuc, pInkB.TkpLuc and pInkC.TkpLuc. In COS-7 cells transfected with the RARα-expression vector, pInk.TkpLuc and pInkA.TkpLuc were induced approximately twofold by retinoic acid (1 μM), whereas pInkB.TkpLuc and pInkC.TkpLuc failed to respond to this treatment (Figure 5b). Under these conditions the transcriptional response to retinoic acid therefore appears to involve the palindromic rather than the direct-repeat pair of half-sites, as the A motif is dispensible. In mobility-shift assays the same requirements were shown for binding of the RAR-containing complex generated in transfected COS-7 cells; mutation of the A motif had little effect, whereas oligonucleotides InkB and InkC bound this complex very weakly or not at all (Figure 5c). Likewise with the HIT-T15-cell nuclear extract little or no binding to the InkB and InkC probes was observed. With single copies of wild-type or mutant Ink oligonucleotides no significant transcriptional response to T_3 has yet been observed, thus we are unable to determine the sequence requirements for transcriptional activation by this ligand.

Finally, we investigated the effect of retinoic acid on expression of the endogenous human insulin gene. There are no available human β-cell lines, so these experiments were performed on isolated human islets of Langerhans. Insulin mRNA levels increased by 4.5- and 4.0-fold after incubation of human islets in retinoic acid for 16 h and 60 h respectively (Figure 6a). In the absence of retinoic acid the insulin mRNA levels remained unchanged over this time period (Figure 6b).

DISCUSSION

The deletion analysis between −1995 and −258 revealed several transcriptionally active regions upstream of the human insulin gene. Some of these, such as the negative regulatory element at −270, have been previously described. A novel finding of this study was the transcriptional effects upon deletion of sequences upstream of the ILPR. Of particular interest was the presence of a hormone-response element located at about 1 kb upstream of the transcription start site (the Ink sequence). We also observed an effect on deletion of the ILPR itself. Although we cannot rule out the presence of regulatory sequences close to the 5′ and 3′ boundaries of the ILPR, our results suggest that the ILPR may play a role in transcription of the human insulin gene.

The Ink sequence closely resembles other sequences that mediate transcriptional responses to both retinoic acid and thyroid hormone. The A–B direct-repeat element with 2 bp spacing (DR-2) is very similar to sequences approx. 1 kb upstream of the mouse and rat CRBP genes [29] and the mouse CRABP gene [30]. These sequences mediate transcriptional activation by retinoic acid, and are believed to bind RAR/RXR heterodimers. The palindromic B–C element is similar to a sequence in the 5′ region of the bovine growth-hormone gene, and is even more closely related to the derived TREpal sequence; both of these demonstrate regulation by retinoic acid and thyroid hormone [31,32]. The rat oxytocin gene contains an element at around −172 to −148 that is responsive to multiple members of the steroid/thyroid receptor superfamily. This element contains a direct repeat of the AGGTCA sequence with zero spacing (DR-0) as well as a palindrome [33]. A further member of the steroid receptor superfamily, the peroxisome-activated receptor protein α (PARPα) is activated by a wide variety of peroxisome proliferators and by fatty acids [34]. PARPα recognizes a direct repeat of the sequence TGACCT in the peroxisomal enzyme acyl-CoA oxidase gene [35]. This site resembles the InkA element on the complementary strand, except that the PARPα element has the structure DR-1 whereas the InkA element has the structure DR-2. The spacing in the PARPα element is important in so far as DR-1 is much more responsive to receptor than DR-2 or DR-0 [34]. This suggests that the InkA element is unlikely to respond to activated PARPα and potentially act as a fatty acid-response element.

In COS-7 cells the A motif of the Ink sequence, which forms part of the direct-repeat element, was dispensible for retinoic acid

regulation, whereas regulation was lost on mutation of the B or the C motif, which together form the palindromic element. In retinoic acid-response elements of the direct-repeat type, the final residue of the AGGTCA consensus is very strongly conserved, but in the InkA motif it is replaced by cytosine. It is possible that this difference renders the A–B direct repeat a poor responder to retinoic acid. In extracts from COS-7 cells overexpressing RARα, the InkA mutant oligonucleotide displayed wild-type binding activity, whereas InkB and InkC showed substantially weaker binding. At present, however, we have not determined whether the complexes detected in this assay contain RAR homodimers or RAR/RXR heterodimers.

The results of the Northern-blot study suggest that the endogenous human insulin gene is regulated by retinoic acid. Our data suggest also that this effect is likely to be mediated through the Ink sequence. When examined within the context of the insulin gene, the Ink sequence responded positively when cells were treated with retinoic acid or transfected with an RAR-expression vector. However, it is interesting that a negative effect was observed when the cells were transfected with the RAR vector and treated with retinoic acid. A similar negative effect of retinoic acid on a retinoic acid-response element in the Oct-3/4 gene has been reported [36,37]. Also, whereas RARα and RXRβ will stimulate the reporter activity driven by a thyrotropin gene construct in transfected COS cells, treatment of the transfected cells with retinoic acid suppresses the activity to 56% of that seen with the receptors alone [38]. It is possible that the activity of the Ink sequence within the larger insulin gene fragment is dependent on the relative concentration of transcription factors, such as helix–loop–helix and octamer-binding proteins, that are known to modulate RAR effects [39]. Such interactions with unrelated transcription factors may permit the activity of the hormone-response element to be modulated positively or negatively in a temporal or cell-specific manner. For example, a part of the S14 gene 5′ region mediates transcriptional induction by retinoic acid in differentiated adipocytes but not in undifferentiated pre-adipocytes, whereas a synthetic retinoic acid-response element is active in both cell types [40]. Similarly, an RXR response element of the CRBPII promoter is activated by retinoic acid and RXR in transfected COS-1, CV-1 and HeLa cells but not in the human colonic carcinoma cell line CaCo-2 under conditions where the activity of the RARα2 gene promoter is induced by retinoic acid [41].

Both *in vivo* and *in vitro* insulin secretion from rat islets is regulated by vitamin A (retinol), a precursor of retinoic acid [42]. In the rat β-like cell line RINm5F, RAR mRNAs can be detected [43–45]. This strongly suggests a role for retinoic acid in the regulation of β-cell function. As retinoic acid frequently participates in developmental regulatory processes [46], it is also conceivable that this ligand is involved in the process of islet differentiation. In fully developed mammalian islets of Langerhans, each of the peptide hormones, i.e. insulin, glucagon, somatostatin and pancreatic polypeptide, is expressed by a specialized cell type (β, α, δ and PP respectively) with little or no co-expression of hormones by a given cell type. It is believed that the four cell types arise during development from common pluripotent precursor cells which at an early stage may co-express two or more peptide hormone genes [47]. The roles of retinoic acid, its cellular binding protein and the Ink sequence in islet cell determination and differentiation remain to be established.

This work was supported by a grant from the Wellcome Trust. A. R. C. was supported by a Fellowship from the British Diabetic Association and M. E. W. by a studentship from the Medical Research Council. We thank G. R. Williams for helpful discussions.

REFERENCES

1 Docherty, K. and Clark, A. R. (1995) in Diabetes: Clinical Science in Practice (Leslie, R. D. G. and Robbins, D., eds.), pp. 15–31, Cambridge University Press, Cambridge
2 Boam, D. S. W., Clark, A. R. and Docherty, K. (1990) J. Biol. Chem. 265, 8285–8296
3 Karlsson, O., Walker, M. D., Rutter, W. J. and Edlund, T. (1989) Mol. Cell. Biol. 9, 823–827
4 Read, M. L., Clark, A. R. and Docherty, K. (1993) Biochem. J. 295, 233–237
5 Read, M. L., Smith, S. B. and Docherty, K. (1995) Biochem. J., in the press
6 Reibel, L., Besnard, C., Lores, P., Jami, J. and Gacon, G. (1993) Nucleic Acids Res. 21, 1595–1600
7 Boam, D. S. W. and Docherty, K. (1989) Biochem. J. 264, 233–239
8 Bell, G. I., Selby, M. J. and Rutter, W. J. (1982) Nature (London) 295, 31–35
9 Hammond-Kosack, M. C. U. and Docherty, K. (1992) FEBS Lett. 301, 79–82
10 Hammond-Kosack, M. C. U., Dobrinski, B., Lurz, R., Docherty, K. and Kilpatrick, M. W. (1992) Nucleic Acids Res. 20, 231–236
11 Hammond-Kosack, M. C. U., Kilpatrick, M. W. and Docherty, K. (1993) J. Mol. Endocrinol. 10, 121–126
12 Hammond-Kosack, M. C. U., Kilpatrick, M. W. and Docherty, K. (1993) J. Mol. Endocrinol. 9, 221–225
13 Fromont-Racine, M., Bucchini, D., Madsen, O. et al. (1990) Mol. Endocrinol. 4, 669–677
14 Bugge, T. H., Pohl, J., Lonnoy, O. and Stunnenberg, H. G. (1992) EMBO J. 11, 1409–1418
15 Zhang, X., Hoffmann, B., Tran, P. B.-V., Graupner, G. and Pfahl, M. (1992) Nature (London) 355, 441–446
16 Yu, V. C., Delsert, C., Andersen, B. et al. (1991) Cell 67, 1251–1266
17 Hallenbeck, P. L., Marks, M. S., Lippoldt, R. E., Ozato, K. and Nikodem, V. M. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 5572–5576
18 Levin, A. A., Sturzenbecker, L. J., Kazmer, S. et al. (1992) Nature (London) 355, 359–361
19 Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A. et al. (1992) Cell 68, 397–406
20 Umesono, K., Murakami, K. K., Thompson, C. C. and Evans, R. M. (1991) Cell 65, 1255–1266
21 Perlmann, T., Rangarajan, P. N., Umesono, K. and Evans, R. M. (1993) Genes Dev. 7, 1411–1422
22 London, N. J. M., James, R. F. L. and Bell, R. F. (1992) in Pancreatic Islet Transplantation (Ricordi, C., ed.), pp. 113–124, R. G. Landis, New York
23 Bell, G. I., Pictet, R. L., Rutter, W. J., Cordell, B., Tischer, E. and Goodman, H. M. (1980) Nature (London) 284, 26–32
24 Clark, A. R., Boam, D. S. W. and Docherty, K. (1989) Nucleic Acids Res. 17, 10130
25 Docherty, K. and Clark, A. R. (1993) in Transcription – A Practical Approach (Hames, B. D. and Higgins, S. J., eds.), pp. 64–123, IRL Press, Oxford
26 Boam, D. S. W., Clark, A. R., Shennan, K. I. J. and Docherty, K. (1990) in Peptide Hormone Secretion (Hutton, J. C. and Siddle, K. eds.), pp. 271–307, Oxford University Press, Oxford
27 Schreiber, E., Matthias, P., Müller, M. M. and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
28 Chomczynski, P. and Saachi, N. (1987) Anal. Biochem. 162, 156–159
29 Smith, W. C., Nakshatri, H., Leroy, P., Rees, J. and Chambon, P. (1991) EMBO J. 10, 2223–2230
30 Durand, B., Saunders, M., Leroy, P., Leid, M. and Chambon, P. (1992) Cell 71, 73–85
31 Bedo, G., Santisteban, P. and Aranda, A. (1989) Nature (London) 339, 231–234
32 Glass, C. K., Holloway, J. M., Devarym, O. V. and Rosenfeld, M. G. (1988) Cell 54, 313–323
33 Adan, R. A. H., Cox, J. J., Beischlag, T. V. and Burbach, J. P. H. (1993) Mol. Endocrinol. 7, 47–57
34 Issemann, I., Prince, R. A., Tugwood, J. D. and Green, S. (1993) J. Mol. Endocrinol. 11, 37–47
35 Tugwood, J. D., Issemann, I., Anderson, R. G., Bundell, K. R., McPhear, W. L. and Green, S. (1992) EMBO J. 11, 433–439
36 Pikarsky, E., Sharir, H, Ben-Shushan, E. and Bergman, Y. (1994) Mol. Cell. Biol. 14, 1026–1038
37 Schoorlemmer, J., van Puijenbroek, A., van den Eijnden, M., Jonk, L., Pals, C. and Kruijer, W. (1994) Mol. Cell. Biol. 14, 1122–1136
38 Breen, J. J., Matsuura, T., Ross, A. C. and Gurr, J. A. (1995) Endocrinology 136, 543–549
39 Lanigan, T. M., Tverberg, L. A. and Russo, A. F. (1993) Mol. Cell. Biol. 13, 6079–6088
40 Lepar, G. J. and Jump, D. B. (1992) Mol. Cell. Endocrinol. 84, 65–72
41 Nakshatri, H. and Chambon, P. (1994) J. Biol. Chem. 269, 890–902
42 Chertow, B. S., Blaner, W. S., Baranetsky, N. G. et al. (1987) J. Clin. Invest. 79, 163–169

43 Kato, M., Kato, K., Blaner, W. S., Chertow, B. S. and Goodman, D. S. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 2488–2492
44 Chertow, B. S., Moore, M. R., Blaner, W. S., Wilford, M. R. and Cordle, M. B. (1989) Diabetes **38**, 1544–1548
45 Chertow, B. S., Blaner, W. S., Rajan, N. et al. (1993) Diabetes **42**, 1109–1114
46 Morriss-Kay, G. (1992) In Cancer Surveys Volume 14: Growth Regulation by Nuclear Hormone Receptors, pp. 181–193, Imperial Cancer Research Fund, London
47 Madsen, O. D., Serup, P., Karlsen, K. et al. (1993) Biochem. Soc. Trans. **21**, 142–146

Received 12 July 1994/24 March 1995; accepted 29 March 1995